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A kinase-phosphatase network that regulates kinetochore-microtubule attachments and the SAC.

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Abstract

The KMN network (for KNL1, MIS12 and NDC80 complexes) is a hub for signalling at the outer kinetochore. It integrates the activities of two kinases (MPS1 and Aurora B) and two phosphatases (PP1 and PP2A-B56) to regulate kinetochore-microtubule attachments and the spindle assembly checkpoint (SAC). We will first discuss each of these enzymes separately, to describe how they are regulated at kinetochores and why this is important for their primary function in controlling either microtubule attachments or the SAC. We will then discuss why inhibiting any one of them individually produces secondary effects on all the others. This cross-talk may help to explain why all enzymes have been linked to both processes, even though the direct evidence suggests they each control only one. This chapter therefore describes how a network of kinases and phosphatases work together to regulate two key mitotic processes.

1. Introduction

The kinetochore is a complex molecular machine consisting of over 100 different proteins (Nagpal and Fukagawa 2016; Pesenti et al. 2016). These proteins can be classified based on whether they perform a structural, functional and/or regulatory role. This broadly divides the kinetochore into three core parts: 1) a constitutive inner network that is involved in tethering the outer kinetochore to chromosomes, 2) a constitutive outer network that reaches out to capture and hold on to microtubules, and 3) a dynamic regulatory set of regulatory components that transiently associate with the kinetochore at the appropriate times. These dynamic components are principally focussed on the KMN network, which is responsible for regulating the kinetochores two main functions: the physical attachment to microtubules and activation of the SAC.

The attachment to microtubules needs to be regulated to ensure that incorrect attachments, which fail to generate tension, can be removed in a process known as error-correction (Krenn and Musacchio 2015). The SAC, on the other hand, must be activated by unattached kinetochores to hold cells in mitosis and give time for tension-generating attachments to form (Joglekar 2016; Lischetti and Nilsson 2015; Musacchio 2015). This chapter focusses on the main kinetochore kinases and phosphatases that regulate these two processes: MPS1, Aurora B, PP1 and PP2A-B56.

At the beginning of mitosis, during prophase, kinase activities predominate at kinetochores: MPS1 phosphorylates proteins that initiate SAC signalling (symbolised by KNL1 in Figure 1A), whereas Aurora B phosphorylates proteins to prevent their attachment to microtubules (symbolised by NDC80 in Figure 1A). Upon nuclear envelope breakdown at the start of prometaphase, however, the phosphatases PP1 and PP2A-B56 are recruited to KNL1 where they begin to counteract the activity of the kinases. This reduces Aurora B activity to allow kinetochore-microtubule attachments to form and primes kinetochores to be ready to silence the SAC (Figure 1B). When these microtubule attachments generate tension, kinase activities are reduced and phosphatase activities predominate, which stabilises these attachments and locally silences the SAC (Figure 1C). When all kinetochores have achieved this stably attached state, the SAC is switched off globally, sister chromatids separate and the cell can exit mitosis.

As will become apparent, although these kinases and phosphatases perform very specific roles at kinetochores, there is also considerable interplay between them: The kinases regulate each other,

the phosphatases regulate each other, and both kinases also regulate, directly or indirectly, both phosphatases (Figure 1). Not surprisingly, this has contributed to a great deal of confusion in the field with regards to "who controls what". We shall attempt to clarify some of this confusion by first discussing the SAC and error-correction processes separately, from the point of view of their direct *bona fide* kinases and phosphatases. This will explain how these enzymes are regulated and why this is important to control either kinetochore-microtubule attachments or the SAC. We will then discuss the issue of cross-talk and explain why manipulating any one of these enzymes produces secondary effects on all the others. This demonstrates that all four enzymes form part of an interconnected network that regulates both mitotic processes.

2. MPS1: The SAC kinase

Monopolar spindle 1 (MPS1) was originally identified as a gene that controls spindle pole body duplication in *Saccharomyces cerevisiae* (Winey et al. 1991), but later shown to be a dual-specificity protein kinase essential for the spindle checkpoint response (Lauze et al. 1995; Poch et al. 1994; Weiss and Winey 1996). The first clues that MPS1 was an upstream component in the checkpoint signalling pathway came with the finding that overexpression of MPS1 alone is sufficient to cause a checkpoint-dependent arrest in *Schizosaccharomyces pombe* in the absence of spindle perturbations (Hardwick et al. 1996). MPS1 was subsequently shown to localise to unattached kinetochores (Abrieu et al. 2001; Castillo et al. 2002; Fisk and Winey 2001; Stucke et al. 2002) where it auto-phosphorylates on key residues in the activation loop and P+1 loop (Kang et al. 2007; Mattison et al. 2007) in *trans* following dimerization (Hewitt et al. 2010). MPS1 kinetochore localisation is critical for SAC activity because N-terminal truncations abolish both kinetochore recruitment and the SAC, and artificially rescuing the localisation is sufficient to reactivate the SAC (Heinrich et al. 2012; Nijenhuis et al. 2013).

It is important to note that this kinetochore localisation is dynamic and MPS1 rapidly exchanges with the cytosol throughout mitosis (Howell et al. 2004; Jelluma et al. 2010). There is no known function of MPS1 in the cytosol, therefore it is tempting to speculate that the activity of MPS1 may be confined to the kinetochore. For example, auto-inhibitory mechanisms may be relieved upon kinetochore binding or active MPS1 may be susceptible to dephosphorylation by cytosolic phosphatases. The development of FRET-based reporters of MPS1 activity would help to shed light on exactly when and where MPS1 is active during mitosis.

When kinetochore-microtubule attachments form, the SAC needs to be silenced and a key event is the removal of MPS1 from kinetochores. Tethering MPS1 to kinetochores, by fusing to the outer kinetochores proteins MIS12 (Jelluma et al. 2010; Heinrich et al. 2012) or NDC80 (Ito et al. 2012), maintains MPS1 on attached kinetochores and prevents SAC silencing. Furthermore, re-recruitment of MPS1 to kinetochores that have already silenced the SAC at metaphase rapidly re-establishes the SAC and arrests mitotic exit (Kuijt et al. 2014; Ballister et al. 2014). This demonstrates that MPS1 recruitment is a key upstream event in SAC signalling, which explains why MPS1 activity is required for the recruitment of all known SAC components to the kinetochore (Heinrich et al. 2012; Santaguida et al. 2010; Kwiatkowski et al. 2010; Maciejowski et al. 2010; Hewitt et al. 2010; Sliedrecht et al. 2010). Furthermore, the fact that loss or gain of kinetochore MPS1 is sufficient to silence or activate the SAC, suggested that MPS1 could be the key "sensor" responsible for detecting kinetochore-microtubule attachment status and relaying this information to the spindle checkpoint machinery. The mechanistic basis for this sensing mechanisms was recently solved.

MPS1 binds directly to the NDC80 complex, the key microtubule attachment site at the outer kinetochore, via the Calponin Homology (CH) domains of NDC80 and NUF2 (Hiruma et al. 2015; Ji et al. 2015). One or both of these CH domains also bind to microtubules (Wilson-Kubalek et al. 2008; Alushin et al. 2010; Ciferri et al. 2008; Sundin et al. 2011), and the MPS1-NDC80 interaction is

inhibited in the presence of microtubules *in vitro* (Hiruma et al. 2015; Ji et al. 2015). The prediction, therefore, is that competition between MPS1 and microtubules is likely to contribute to SAC silencing following stable kinetochore-microtubule attachment *in vivo*. It is important to note that although this mechanism was uncovered in humans, it may not have been conserved throughout evolution. MPS1 does not need to be removed from kinetochores to silence the SAC in budding yeast. Instead, a change in kinetochore structure following microtubule attachment is sufficient to spatially restrict access of MPS1 to its key kinetochore substrate, KNL1 (Aravamudhan et al. 2015; Joglekar 2016). In fact, others have argued that this may also be how MPS1 signalling is silenced in human cells (Musacchio 2015). The argument that it could at least contribute is valid and warrants further investigation.

An additional mechanism that could contribute to SAC silencing relates to inhibition of any one of the upstream inputs needed for MPS1-NDC80 interaction. Phosphorylation of MPS1 or NDC80 (by either Aurora B, MPS1 or CDK1) has been shown to enhance MPS1-NDC80 binding *in vitro* (Hiruma et al. 2015; Ji et al. 2015). Therefore, if phosphorylation is required for MPS1 kinetochore localisation *in vivo*, then a reduction in any one of these inputs following microtubule attachment/tension could contribute to MPS1 removal and SAC silencing. In this regard, Aurora B activity could well be the most relevant, because it is required for the localisation of MPS1 to kinetochores in the absence of microtubules (Jelluma et al. 2010; Santaguida et al. 2010; Saurin et al. 2011), and tension across kinetochores is known to reduce localised Aurora B activity (Liu et al. 2009). Furthermore, Aurora B-mediated phosphorylation of the NDC80 tail region, which is lost as microtubules make stable attachments with kinetochores, has been directly implicated in MPS1-NDC80 interaction *in vitro* (Ji et al. 2015) and MPS1 localisation *in vivo* (Zhu, 2013 #497). Others have questioned the validity of the *in vivo* data (Etemad and Kops 2016), therefore, it will be important to clarify whether Aurora B controls MPS1 localisation directly *in vivo* and, if so, whether this occurs via NDC80 phosphorylation or alternative mechanisms.

The importance of MPS1 activity for its own localisation is still puzzling: MPS1 auto-phosphorylation enhances NDC80 interaction *in vitro* (Hiruma et al. 2015), and yet MPS1 inhibition increases kinetochore accumulation in cells (Jelluma et al. 2010). The reason for this difference is not clear, but the enhanced localisation in cells may result from feedback that is missing *in vitro*. A potential explanation could be provided by the recent discovery that ARHGEF17 controls MPS1 kinetochore localisation (Marquardt and Fisk 2016). MPS1 phosphorylates ARGEF17 to drive its own release from kinetochores, therefore MPS1 inhibition preserves kinetochore localisation in cells. It will be important in future to determine how ARGEF17 targets MPS1 to kinetochores (i.e. via NDC80 or not) and whether this is regulated directly by Aurora B.

The initiation of SAC downstream of MPS1 will be discussed in detail in an accompanying chapter of this book. It is also the subject of some excellent recent reviews (Joglekar 2016; Lischetti and Nilsson 2015; Musacchio 2015). To understand the remainder of this chapter, however, it is important to state that a key event downstream of MPS1 is the phosphorylation of KNL1 on 'MELT' repeats (Figure 1B) (Yamagishi et al. 2012; London et al. 2012; Shepperd et al. 2012). This KNL1 phosphorylation initiates a cascade of events that culminate in the assembly of the mitotic checkpoint complex, which can diffuse throughout the cytoplasm to inhibit APC-CDC20 and prevent mitotic exit (Izawa and Pines 2015).

3. Aurora B: The error-correction kinase

Aurora B is a member of the Aurora family of serine/threonine protein kinases, originally discovered in yeast (Chan and Botstein 1993) and later found to regulate chromosome segregation in several species including human (Glover et al. 1995; Gopalan et al. 1997; Kimura et al. 1997; Biggins et al. 1999; Hauf et al. 2003; Ditchfield et al. 2003). The main role of Aurora B in chromosome segregation

is to regulate attachments between kinetochores and microtubules (Tanaka et al. 2002; Biggins et al. 1999; Hauf et al. 2003; Lampson et al. 2004); although it also has important roles in regulating sister chromatid cohesion (Losada et al. 2002; Nishiyama et al. 2013; Kim et al. 2013; Dai et al. 2006; Gimenez-Abian et al. 2004), assembling the outer kinetochore (Dimitrova et al. 2016; Petrovic et al. 2016; Kim and Yu 2015; Rago et al. 2015), and regulating the SAC (as discussed later) (Maldonado and Kapoor 2011; Santaguida et al. 2011; Saurin et al. 2011; Vader et al. 2007).

The principle behind its role in regulating attachments is that Aurora B can selectively destabilise microtubules from kinetochores that are incorrectly attached, whilst leaving correctly attached microtubules intact (Figure 2). Therefore, although the chromosome attachment process is error-prone, the presence of Aurora B allows these errors to be corrected in iterative cycles of detachment and attachment until tension is achieved and kinetochore fibres remain stably bound. This immediately raises two important questions: how does Aurora B detach kinetochore-microtubules and how is that detachment process regulated by tension?

The mechanisms by which Aurora B destabilises microtubule attachments is now well understood: it phosphorylates and inhibits key proteins at the outer kinetochore that are involved in stabilising those attachments. The NDC80 complex is one of the main outer kinetochore proteins that interacts with microtubules, via its CH domain and N-terminal tail, and Aurora B phosphorylates up to nine residues within this tail region to electrostatically interfere with microtubule binding (Miller et al. 2008; Guimaraes et al. 2008; Ciferri et al. 2008; DeLuca et al. 2006; Cheeseman et al. 2006; Alushin et al. 2010; Wei et al. 2007). In addition to simply binding microtubules, the kinetochore must also hold onto these microtubules as they depolymerise. This is believed to be dependent on two structurally unrelated, but functionally homologous, complexes in budding yeast (DAM1) or higher eukaryotes (SKA), both of which are phosphorylated by Aurora B to prevent microtubule interaction and/or kinetochore association (Asbury et al. 2006; Westermann et al. 2006; Welburn et al. 2009; Tanaka et al. 2007; Raaijmakers et al. 2009; Hanisch et al. 2006a; Gaitanos et al. 2009; Cheeseman et al. 2002; Chan et al. 2012; Schmidt et al. 2010). Finally, Aurora B also phosphorylates additional outer kinetochore components (Welburn et al. 2010; Hua et al. 2011), microtubule binding proteins (Iimori et al. 2016), and microtubule-depolymerising kinesins (Zhang et al. 2007; Ohi et al. 2004; Knowlton et al. 2009; Lan et al. 2004; Andrews et al. 2004) to fine-tune the stability of microtubule attachments. Therefore, Aurora B phosphorylates multiple targets at the outer kinetochore to destabilise microtubule attachments, but why does it do this preferentially for kinetochore-microtubules that fail to generate tension? The short answer is that the activity of Aurora B is higher at these tension-less kinetochores (Liu et al. 2009). To explain the theories as to why, we must first explain how Aurora B activity and localisation is controlled.

Aurora B is part of the chromosome passenger complex (CPC), which also contains the Inner Centromere Protein (INCENP), Survivin and Borealin (Jeyaprakash et al. 2007). The Survivin and Borealin subunits are anchored to the N-terminus of INCENP, whereas the catalytic activity of the complex is provided by the Aurora B subunit bound to the C-terminus of INCENP (Krenn and Musacchio 2015). Activation of this catalytic subunit is controlled by multiple steps. First, a short region in the C-terminus of INCENP (termed the IN-box) binds near the active site to stimulate low levels of kinase activity. This allows subsequent auto-phosphorylation of the IN-box (on a TSS motif) and Aurora B itself (within the activation loop), which stimulates full activity of the complex (Sessa et al. 2005; Honda et al. 2003; Bishop and Schumacher 2002,Yasui, 2004 #534). Auto-phosphorylation of at least the TSS motif is believed to occur in *trans* (Zaytsev et al. 2016; Sessa et al. 2005), which explains why artificial clustering of the CPC *in vitro* (Kelly et al. 2007) or *in vivo* (Wang et al. 2011a) activates Aurora B. This also explains why the endogenous pathways that promote CPC clustering in cells are so important for Aurora B activation.

At the start of mitosis the CPC initially clusters on chromatin before it concentrates at the centromere (Carmena et al. 2012b). This centromeric localisation pathway is controlled by multiple different kinase and phosphatase feedback loops that converge on key phospho-dependent interactions between histone tails and the Borealin or Survivin subunits of the CPC. In short, Histone H3-Thr3 (H3-T3) phosphorylation by Haspin creates a docking site for Survivin (Jeyaprakash et al. 2011; Kelly et al. 2010; Wang et al. 2010; Yamagishi et al. 2010; Niedzialkowska et al. 2012) and H2A-Thr120 (H2A-T120) phosphorylation by BUB1 allows the binding of Shugoshin proteins (SGO1 or SGO2), which also interact with CDK1-phosphorylated Borealin (or Survivin in fission yeast) (Kawashima et al. 2007; Kawashima et al. 2010; Yamagishi et al. 2010; Tsukahara et al. 2010). Aurora B activity reinforces its own localisation by activating Haspin (Ghenoiu et al. 2013; Wang et al. 2011b), promoting the kinetochore localisation of BUB1 (Vigneron et al. 2004), and inhibiting the chromatin localisation of the H3-T3 phosphatase RepoMan-PP1 (Qian et al. 2013). These pathways rapidly converge on the centromere because kinetochore-BUB1 phosphorylates H2A-T120 on histone tails in the centromeric region, and the H3-T3 kinase Haspin binds cohesin rings (Yamagishi et al. 2010), which are themselves concentrated at the centromere by a pathway involving BUB1/H2A-T120/SGO1 in human cells (Haarhuis et al. 2014). In addition, the inner centromeric recruitment of Haspin has recently been shown to be regulated by interaction with a SUMOylated form of Topoisomerase II (Edgerton et al. 2016; Yoshida et al. 2016).

The role of this centromeric CPC recruitment is to stimulate Aurora B activation by promoting *trans*-autophosphorylation, and to position this active pool close enough to the outer kinetochore to regulate microtubule attachment. The fact that Aurora B is 'close' but not 'at' the outer kinetochore is critical for the ability of the CPC to sense tension. The basic principle here is that the active pool of Aurora B is in range of its key substrates at kinetochores, but when tension is applied by microtubules, these substrates are pulled away from this zone of activity (Tanaka et al. 2002; Liu et al. 2009). Although the importance of the centromeric pool of Aurora B has recently been questioned (Campbell and Desai 2013), the principle of "spatial separation" is still a widely-accepted model for tension-sensing. Exactly how this process works, however, is still very much a matter of debate (Krenn and Musacchio 2015; Lampson and Cheeseman 2011).

The debate principally concerns how Aurora B reaches its substrates from the centromere and why this is restricted by the tension exerted by microtubules. Aurora B could potentially reach from its anchor point at the centromere, assuming INCENP acts as a flexible linker to provide the required length. In this 'leash' model, small changes in distance could physically pull the kinetochore out of reach of the Aurora B subunit (Santaguida and Musacchio 2009; Maresca and Salmon 2010). A simple prediction is that reducing the length of the leash should disturb error correction, however chromosome alignment is unaffected by deletion of the INCENP coiled-coil region (Vader et al. 2007). Alternatively, Aurora B could defuse away from the centromere to reach its outer kinetochore targets (Wang et al. 2011a). However, as pointed out previously by others (Krenn and Musacchio 2015), it is unlikely that simple diffusion alone could account for the very sharp cut-off in Aurora B activity that occurs within the 100nm length scale of the kinetochore. At least part of the answer could be explained by the presence of phosphatases to sharpen this gradient near kinetochores.

Using a coupled in vitro kinase-phosphatase system, Aurora B was shown to exist in distinct high and low activity states, which may contribute to the required switch-like behaviour at kinetochores (Zaytsev et al. 2016). The role of phosphatase regulation in this regard is perhaps particularly important. Both PP1 and PP2A-B56 localise to the outer kinetochore and their regulated localisation and/or activity could contribute to the inactivation of kinetochore Aurora B or its substrates following microtubule attachment/tension (as discussed later). In fact, the possibility that tension may alter kinetochore phosphatase activity could potentially unite two camps that currently disagree over the tension-sensing mechanism (Krenn and Musacchio 2015; Campbell and Desai

2013). The debate principally concerns whether the important tension-dependent changes are intrinsic or extrinsic to the kinetochore. If intrinsic phosphatase activation worked together with an extrinsic increase in distance (to produce a sharp Aurora B gradient near kinetochores, for example), then both mechanisms could in fact contribute to tension-sensing. The important distance changes may depend on centromere-kinetochore distance, as originally proposed, or on the distance between the outer kinetochore and other pools of Aurora B that may reside in or around kinetochores. Furthermore, these ideas need not be mutually exclusive either, since these 'other pools' could also indirectly depend on centromeric Aurora B for activity: The CPC could potentially use interactions with microtubules to position itself near to kinetochores (Banerjee et al. 2014; Krupina et al. 2016; Campbell and Desai 2013), or it may interact with components within the kinetochore itself. Although the existence of these kinetochore interaction interfaces is still speculative, it is important to note that several groups have reported a significant pool of active Aurora B that resides in or near the outer kinetochore (using the phospho-Aurora B-Thr232 activation loop antibody) (Posch et al. 2010; DeLuca et al. 2011; Caldas et al. 2013). How this active pool is regulated is unknown, but it may require the N-terminus of KNL1 (Caldas et al. 2013) and/or CPC dimerization (Bekier et al. 2015). It will be important in future to clarify whether this truly reflects an active pool of Aurora B (and not simply a cross-reacting Aurora B substrate) and, if it does, to determine whether this active pool is regulated by tension.

Finally, it is also important to note that microtubule attachment/tension is likely to feedback onto Aurora B and regulate its localisation to the centromere. Unaligned kinetochores have higher centromeric Aurora B levels than aligned kinetochores, which can be explained by the tension-sensitive removal of Sgo1/2 due to the separation of kinetochore BUB1 away from its centromeric substrate Histone H2A (Salimian et al. 2011; Tanno et al. 2015; Nerusheva et al. 2014). Therefore, tension is likely to reduce the zone of Aurora B activity that originates from centromeres, in addition to separating the outer kinetochore away from this zone of activity (Figure 2).

4. The antagonising phosphatases

Having discussed the main kinases that regulate the SAC and kinetochore-microtubule attachments, we will now discuss the phosphatases that antagonise these kinase inputs. The first evidence that a phosphatase was required for chromosome segregation came from genetic screens in fission yeast, which implicated protein phosphatase 1 (PP1) homologues in chromosome disjunction and mitotic exit (Ohkura et al. 1988; Booher and Beach 1989; Ohkura et al. 1989). Clues as to the molecular targets of PP1 came from subsequent work in budding yeast that showed how mutation of *glc7*, the PP1 homologue, can partially suppress mutation in *ipl1*, the Aurora B homologue (Francisco et al. 1994). This antagonism between Aurora B and PP1 was later demonstrated for the Aurora B substrate on chromatin, Histone-H3 (pSer10) (Murnion et al. 2001; Hsu et al. 2000). Although this explained why the chromatin-association of PP1 peaks during anaphase, when the bulk of Histone H3 is rapidly dephosphorylated, it did not explain why inhibiting PP1 caused a mitotic arrest much earlier in prometaphase. Furthermore, this mitotic arrest was associated with unstable kinetochore-microtubule attachments and persistent SAC activation, implying that the relevant PP1 substrates may reside at the kinetochore (Sassoon et al. 1999; Bloecher and Tatchell 1999). PP1 was subsequently confirmed as a kinetochore-localised phosphatase that peaks during metaphase, when kinetochore substrates that regulate either microtubule attachment or the SAC need to be dephosphorylated (Trinkle-Mulcahy et al. 2003; Alvarez-Tabares et al. 2007; Trinkle-Mulcahy et al. 2006; Liu et al. 2010). Not surprisingly, PP1 was soon implicated in both of these processes.

The regulation of the SAC appears to be principally centred on the localised recruitment of PP1 to KNL1, which is a hub for SAC signalling at the kinetochore (Caldas and DeLuca 2014; Ghongane et al. 2014). PP1 associates with the N-terminus of KNL1 via short linear motifs (SILK and RVSF) (Liu et al. 2010; Rosenberg et al. 2011; Meadows et al. 2011; Espeut et al. 2012), which mediate the targeting

of PP1 to many different regulatory subunits (Meiselbach et al. 2006; Hendrickx et al. 2009; Wakula et al. 2003; Egloff et al. 1997). This KNL1 interaction positions PP1 next to the MELT repeats, which are phosphorylated by MPS1 to establish SAC signalling at kinetochores (Yamagishi et al. 2012; London et al. 2012; Shepperd et al. 2012). This pool of PP1 is critical for dephosphorylating these MELT repeats and silencing the SAC in many different species, including humans (Pinsky et al. 2009; Rosenberg et al. 2011; Meadows et al. 2011; Espeut et al. 2012; Vanoosthuyse and Hardwick 2009; Nijenhuis et al. 2014). Although this KNL1-PP1 complex is the best validated PP1 complex at kinetochores, it is important to note that PP1 does bind to other kinetochore proteins as well (Hafner et al. 2014; Akiyoshi et al. 2009; Meadows et al. 2011; De Wever et al. 2014; Tang and Toda 2015; Kim et al. 2010; Sivakumar et al. 2016), some of which have also been implicated in SAC silencing (Meadows et al. 2011; Tang and Toda 2015; Sivakumar et al. 2016). It is also important to point out however, that in human cells at least, these other pools of PP1 cannot silence the SAC when MPS1 is inhibited in the absence of microtubules, because specific mutation of the RVSF motif in KNL1 abolishes SAC silencing under these conditions (Nijenhuis et al. 2014). However, the fact that some of these alternative pools of PP1 are delivered to kinetochores by microtubules suggests that they could aid in recruiting PP1 and silencing the SAC following microtubule attachment.

These data demonstrate that PP1 antagonises MPS1 to shut down the SAC, but does it also play a role in opposing Aurora B at the kinetochore? The early data in yeast certainly implies that it does, because here PP1 balances Aurora B activity to allow chromosome segregation (Francisco et al. 1994; Sassoon et al. 1999; Bloecher and Tatchell 1999; Pinsky et al. 2006). In human cells, however, although KNL1-PP1 does antagonise Aurora B at kinetochores, it only appears to do this following biorientation of those kinetochores (i.e. upon alignment to the metaphase plate) (Liu et al. 2010). Inhibiting KNL1-PP1 interaction destabilises established kinetochore fibres at metaphase, but it does not cause obvious defects in chromosome alignment. This implies that the main role of KNL1-PP1 is to keep Aurora B activity supressed on kinetochores with bipolar (amphitelic) microtubule attachments. Aurora B activity also needs to be counteracted earlier to allow initial microtubule attachments to form. However, in human cells at least, this is the job of a secondary phosphatase - protein phosphatase 2A-B56 (PP2A-B56).

PP2A-B56 localises to unattached kinetochores via its regulatory B56 subunit, and siRNA-mediated depletion of all B56 isoforms causes a severe defect in kinetochore-microtubule attachment (Foley et al. 2011). Although PP2A-B56 has a well-established role in preserving cohesion at centromeres (Marston 2015), this attachment phenotype was unrelated to premature sister chromatid splitting, perhaps because siRNA-mediated depletion is unable to reduce total PP2A-B56 levels below the threshold required to maintain cohesion. Instead, the phenotype of PP2A-B56 depletion is associated with elevated kinetochore Aurora B activity, and inhibition of Aurora B is sufficient to rescue kinetochore-microtubule attachments (Foley et al. 2011). This was reminiscent of the phenotype seen following BUBR1 depletion (Lampson and Kapoor 2005), and subsequent work explained exactly why PP2A-B56 and BUBR1 are functionally linked.

PP2A-B56 localises to kinetochores by binding directly to BUBR1 on its kinetochore attachment regulatory domain (KARD) (Suijkerbuijk et al. 2012; Kruse et al. 2013; Xu et al. 2013). This interaction is strengthened by CDK1 and PLK1-mediated phosphorylation of the KARD, although recent structural and biochemical work suggests that CDK1 phosphorylation is the most functionally important (Hertz et al. 2016; Wang et al. 2016). Inhibiting kinetochore-PP2A-B56 directly, by mutating or deleting the KARD, elevates kinetochore Aurora B activity and disrupts kinetochore-microtubule attachments, which can be rescued by inhibiting Aurora B (Suijkerbuijk et al. 2012; Xu et al. 2013). The conclusion, therefore, is that PP2A-B56 is needed at kinetochores to supress Aurora B activity and allow initial kinetochores-microtubules attachments to form. Once these attachments

have formed correctly, PP2A-B56 is removed and PP1 is recruited, which could explain why PP1 is important to continue inhibiting Aurora B at this time.

These data therefore suggest that kinetochore PP1 and PP2A-B56 can be separated by time, if not by function. PP2A-B56 is needed to balance Aurora B activity on kinetochores prior to their alignment, whereas PP1 takes over to switch off the SAC and stabilise attachments after alignment. This handover is controlled by a switch in the phosphorylation state of the relevant recruitment motifs. The SILK and RVSF motifs in KNL1 are phosphorylated by Aurora B to inhibit PP1 binding (Liu et al. 2010), whereas, as discussed before, the KARD domain in BUBR1 is phosphorylated by CDK1/PLK1 to enhance PP2A-B56 interaction (Suijkerbuijk et al. 2012; Kruse et al. 2013; Xu et al. 2013; Hertz et al. 2016; Wang et al. 2016). Furthermore, BUBR1 itself is recruited to kinetochores by MPS1-dependent MELT phosphorylation (Yamagishi et al. 2012; London et al. 2012; Shepperd et al. 2012). Therefore, on unattached kinetochores, kinases help to elevate PP2A-B56 (MPS1, PLK1 and CDK1) and inhibit PP1 (Aurora B). Following stable microtubule attachment, however, many of these activities are lost, which removes kinetochore-PP2A and increases PP1. As discussed previously, this rise in kinetochore PP1 could also be aided by the localised delivery of extra pools of PP1 down microtubules (Hafner et al. 2014; Meadows et al. 2011; De Wever et al. 2014; Tang and Toda 2015; Kim et al. 2010; Sivakumar et al. 2016).

A final issue regarding phosphatase regulation, which is still relatively poorly understood, is whether catalytic activity can be regulated directly. In this regard, CDK1 activity may be particularly important. CDK1 phosphorylation has been shown to inhibit PP1 isoforms directly (Yamano et al. 1994; Kwon et al. 1997; Wu et al. 2009; Dohadwala et al. 1994; Grallert et al. 2015) and activate a kinetochore inhibitor of PP2A-B56 (Porter et al. 2013). CDK1 specifically localises to unattached kinetochores (Chen et al. 2008), therefore its removal upon microtubule attachment could contribute to the activation of kinetochore phosphatases at this time. It will be important in future to determine whether CDK1 activity is regulated at kinetochores and, if so, whether this impacts on localised phosphatase activation.

5. Signalling cross-talk at kinetochores

5.1. Feedback between kinases

So far, we have presented two linear pathways to explain how the SAC is activated by MPS1 and how kinetochore-microtubule attachments are regulated by Aurora B. MPS1 phosphorylates KNL1 to recruit, directly or indirectly, all SAC components to the kinetochore. Aurora B phosphorylates outer kinetochore proteins to destabilise microtubule attachments. This explains why the SAC is particularly sensitive to MPS1 inhibition, whereas chromosomal alignment is particularly sensitive to Aurora B inhibition. This is an over-simplified picture, however, and there is considerable evidence of cross-talk between these two pathways. MPS1 knockdown or inhibition causes delays in chromosome alignment, which are associated with defective kinetochore-microtubule attachments (Jelluma et al. 2008; Kwiatkowski et al. 2010; Maciejowski et al. 2010; Maure et al. 2007; Santaguida et al. 2010; Sliedrecht et al. 2010). Aurora B knockdown or inhibition causes defects in the SAC, even in the absence of microtubules, which suggests that Aurora B can activate the SAC directly (Maldonado and Kapoor 2011; Vader et al. 2007; Saurin et al. 2011; Santaguida et al. 2011). It is important to note that these phenotypes are considerably milder than those observed following inhibition of the *bona fide* upstream kinase. For example, Aurora B inhibition only sensitizes the SAC (Saurin et al. 2011; Santaguida et al. 2011), whereas MPS1 inhibition overrides it completely (Hewitt et al. 2010; Kwiatkowski et al. 2010; Maciejowski et al. 2010; Santaguida et al. 2010; Sliedrecht et al. 2010). MPS1 inhibition only delays chromosome alignment (Hewitt et al. 2010), whereas Aurora B inhibition abolishes it completely (Ditchfield et al. 2003; Hauf et al. 2003). The obvious conclusion, therefore, is that MPS1 and Aurora B principally regulate the SAC and microtubule attachments,

respectively, but they also have secondary effects that impinge on each other's pathways. What then, could be the molecular basis for this cross-talk?

As discussed previously, the localisation of Aurora B and MPS1 to centromeres and kinetochores is required for their initial activation during mitosis. Aurora B activity is needed to recruit MPS1 to kinetochores, and inhibiting Aurora B causes delays in activating MPS1 and establishing the SAC (Jelluma et al. 2010; Saurin et al. 2011; Santaguida et al. 2011). Similarly, MPS1 activity is needed to recruit Aurora B to centromeres, and MPS1 inhibition delays Aurora B activation and induces defects in kinetochore-microtubule attachment (van der Waal et al. 2012). Therefore, both kinases function in a positive feedback loop that contributes to their switch like activation upon mitotic entry (Figure 3A). This feedback also underlies much of the cross-talk between these two pathways, because uncoupling the feedback also uncouples the cross-talk. For example, recovering MPS1 at kinetochores (using a MIS12 fusion) bypasses any requirement for Aurora B activity in MPS1 activation and the SAC (Jelluma et al. 2010; Saurin et al. 2011). Conversely, recovering Aurora B at centromeres (using a CENPB-INCENP fusion) rescues most of the chromosome alignment defects seen following MPS1 inhibition (van der Waal et al. 2012).

These data suggest that the primary function of Aurora B in the SAC is to localise MPS1 to kinetochores, or alternatively, if Aurora B has additional functions, then these can be bypassed by artificial MPS1 recruitment (as discussed later). Furthermore, defective kinetochore-microtubule attachment upon MPS1 inhibition can be at least partially explained by the mislocalisation of Aurora B. There may well be additional effects of MPS1 inhibition, because restoring Aurora B to centromeres causes an incomplete, albeit substantial, rescue in chromosome alignment (van der Waal et al. 2012). This could reflect abnormal levels or turnover of Aurora B at CENP-B (i.e. Aurora B is rescued at centromeres but not to the exact wild type situation). Alternatively, MPS1 could have additional effects that are either independent of Aurora B activity (Maure et al. 2007; Espeut et al. 2008; Storchova et al. 2011) or Aurora B localisation (Bourhis et al. 2009; Jelluma et al. 2008). These additional effects may become more dominant when MPS1 is inhibited after mitotic entry, because this has minimal impact on Aurora B localisation (van der Waal et al. 2012), but still causes significant defects in kinetochore-microtubule attachment (Sliedrecht et al. 2010; Hewitt et al. 2010).

5.2. Feedback between phosphatases

The kinetochore recruitment of PP1 and PP2A-B56 is regulated by multiple kinases: phosphorylation enhances kinetochore-PP2A-B56 and decreases kinetochore-PP1, therefore, by inference, dephosphorylation must decrease PP2A-B56 and increase PP1. This regulation is also critically dependent on cross-talk. PP1 dephosphorylates the KNL1-MELT and BUBR1-KARD motifs to decrease PP2A-B56, whereas PP2A-B56 dephosphorylates the KNL1-SILK and RVSF motifs to increase PP1 (Figure 3B) (Nijenhuis et al. 2014). Therefore, both phosphatases are engaged in a negative feedback loop that regulates their own localisation to kinetochores. It is therefore important to be cautious when interpreting results following direct phosphatase inhibition, because inhibiting kinetochore PP2A-B56 also inhibits KNL1-PP1, whereas inhibiting KNL1-PP1 increases kinetochore PP2A-B56. Therefore, to untangle direct from indirect effects, it is important to compare the effects of inhibiting *both* phosphatases individually. This is best illustrated using the example of SAC regulation in human cells.

As discussed earlier, PP1 is well known to silence the SAC in many different species, from yeast to worms (Pinsky et al. 2009; Rosenberg et al. 2011; Meadows et al. 2011; Espeut et al. 2012; Vanoosthuyse and Hardwick 2009). Therefore, the discovery that PP2A-B56 is essential for SAC silencing in humans, on the fact of it, appears surprising (Espert et al. 2014; Nijenhuis et al. 2014). However, a close comparison of mutants that interfere with either kinetochore PP2A-B56 or KNL1-

PP1 demonstrated that both phosphatase complexes are essential (Nijenhuis et al. 2014). In fact, the phenotypes are identical, except for one crucial difference: In both scenarios KNL1-PP1 is lost, but when targeting KNL1-PP1 directly, kinetochore PP2A-B56 is actually increased (due to the feedback, see Figure 3B). Therefore, this PP2A-B56 pool cannot silence the SAC, which confirms that KNL1-PP1 is the direct SAC phosphatase in human cells, as it is in lower eukaryotes. PP2A-B56 is only essential to antagonise Aurora B and recruit PP1, because if Aurora B is inhibited then PP2A-B56 is no longer required (Nijenhuis et al. 2014).

In summary, the fact that these two phosphatases control each other's localisation to kinetochores makes it very difficult to tease apart their individual effects. It is particularly important to test carefully whether the effects of PP2A-B56 loss occurs directly, or indirectly, via PP1. Considering that PP1 and PP2A-B56 have both been shown to balance Aurora B and regulate kinetochore-microtubule attachments, it will be important to address the possible role of cross-talk here as well.

Finally, the phosphatase cross-talk discussed so far is based entirely on localisation, even though, as mentioned previously, both phosphatases can be inhibited directly by phosphorylation. It is possible therefore, that they could also reverse these phosphorylations and activate each other directly. In this regard, the recent discovery that $PP1^{Dis2}$ and $B56^{Par1}$ are reactivated sequentially upon mitotic exit in fission yeast is particularly interesting (Grallert et al. 2015). In this situation, $PP1^{Dis2}$ is able to auto-activate and then activate $B56^{Par1}$, both via dephosphorylation. It is hard to reconcile these findings with the fact that both of these enzymes need to be active much earlier in mitosis to function at kinetochores. One possible explanation is that they are globally inhibited by phosphorylation, but then locally reactivated exactly when and where they are needed. In this scenario, kinetochore clustering may elevate phosphatase activity enough to permit localised phosphatase reactivation when required. It will be important in the future to determine whether these phosphatases are indeed inhibited directly during mitosis, and if so, whether they are reactivated in specific locations via cross-talk.

5.3. Kinase-phosphatase cross-talk

Having discussed the feedback between pairs of kinases or phosphatases, we would like to now finish by highlighting the cross-talk that exists between all four of these enzymes. In particular, we would like to discuss how kinases could mediated their effects via the phosphatases (Figure 3C). This is important because it is very easy to adopt a kinase-centric view of signalling, even though phosphatase activity could be equally important. To highlight this point, let us now consider the role of Aurora B in SAC signalling.

As discussed previously, Aurora B activity is required to localise MPS1 to kinetochores and establish the SAC (Jelluma et al. 2010; Saurin et al. 2011; Santaguida et al. 2011). Although the molecular mechanism is still unclear, Aurora B is thought to control kinetochore MPS1 by phosphorylating either NDC80, MPS1, or some other protein required for MPS1-NDC80 interaction. Aurora B is well known to inhibit the phosphatase that antagonises MPS1 and the SAC (KNL1-PP1) (Liu et al. 2010), therefore the input of Aurora B could be wholly, or at least partially, explained by the inhibition of this phosphatase complex (Figure 3C). KNL1-PP1 antagonises MPS1 at the level of MELT phosphorylation, and it may also inhibit MPS1 localisation and/or activity directly. Rescuing MPS1 localisation to kinetochores (with a MIS12-fusion), which bypasses any requirement for Aurora B in the SAC (Saurin et al. 2011), could also potentially rescue all these phosphatase-mediated effects (especially if kinetochore-MPS1 levels are artificially high). It will therefore be important in future to determine just how much of Aurora B's input into the SAC is mediated via KNL1-PP1.

It is important to also consider whether the effects of MPS1 inhibition could also be mediated, at least in part, via the phosphatases. MPS1 activity enhances the kinetochore localisation of PP2A-B56,

via MELT phosphorylation, which can inhibit Aurora B substrates (Suijkerbuijk et al. 2012; Kruse et al. 2013; Xu et al. 2013) and Aurora B directly (Meppelink et al. 2015) (Figure 3C). MPS1 also localises the acetyltransferase TIP60 to kinetochores, which is able to acetylate Aurora B and protect it from PP2A-mediated inactivation (Mo et al. 2016). It is therefore difficult to predict how MPS1 inhibition may impact on the PP2A-B56/Aurora B balance at kinetochores, but this is important to test because this balance is critical for regulating kinetochore-microtubule attachments.

5.4. Cross-talk with other kinases

Finally, we have deliberately focussed this chapter on the principle kinases that regulate the SAC or microtubule attachment, simply to avoid confusion. Other kinases, however, are certainly important. PLK1 activity, for example, is needed to regulate the SAC (von Schubert et al. 2015; O'Connor et al. 2015) and kinetochore-microtubule attachments (Sumara et al. 2004; Hanisch et al. 2006b; Peters et al. 2006; Lenart et al. 2007; Liu et al. 2012). It is also regulated by cross-talk with all four of the enzymes discussed above. PLK1 can phosphorylate MPS1 substrates directly (von Schubert et al. 2015; Espeut et al. 2015), MPS1 itself to inhibit kinetochore-localisation (von Schubert et al. 2015), the KNL1-MELT and BUBR1-KARD motifs to recruit PP2A-B56 (Suijkerbuijk et al. 2012; Kruse et al. 2013; Xu et al. 2013; von Schubert et al. 2015), and the PP1 regulator SDS22 to inhibit the dephosphorylation and inactivation of Aurora B (Duan et al. 2016). Furthermore, PLK1 is itself activated and recruited to kinetochores by Aurora B (Shao et al. 2015; O'Connor et al. 2015; Carmena et al. 2012a) and inhibited and delocalised by PP2A-B56 (Foley et al. 2011). It will clearly be important in future to integrate PLK1 signalling into the complex network of other signals highlighted above.

6. Summary

We have learnt a great deal over the past few decades about how chromosome segregation is regulated. A key aspect of that concerns the kinetochore and, in particular, how this integrates the wide variety of signals needed to safeguard the microtubule attachment process. We now understand most of the enzymes that generate these signals, how these enzymes are regulated, and how they sense and transmit information to the chromosome segregation machinery. Perhaps all that remains is to learn how all this information is integrated together in a way that ensures chromosome segregation can proceed accurately and reliably. This 'final frontier', however, may turn out to be the most challenging of all.

It is becoming increasingly clear that the more we learn about the connections between these signals the more confusing the situation becomes. The four main regulators highlighted here are embedded in a network that is, quite literally, entangled in cross-talk. It is hard for us to predict, even now, why all this cross-talk exists, so how will it feel when new connections are identified or, worse still, when new signals are integrated. The short section on PLK1 may provide a glimpse of the answer.

So, how will we rise to this challenge and what will it take to make the next big step? In the words of the late, great Richard Feynman: "What I cannot create, I do not understand." Or to put this another way, reductionist biology will only take us so far. Building the kinetochore may seem like a mammoth task, but there has been some remarkable recent progress in this area (Pesenti et al. 2016; Weir et al. 2016). There is also the alternative, but equally valid, approach of purifying kinetochores that could then be stripped of their regulatory components (Gonen et al. 2012; Akiyoshi et al. 2010). Reconstituting these *in vitro* systems with a defined set of enzymes would allow us to "create" the signalling network from scratch. If this could be allied with computational approaches to help us comprehend the systems-level behaviour *in vitro*, and then predict the behaviour *in vivo*, perhaps we will then be in a position to claim that we truly "understand".

7. References

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Figure Legends

Figure 1. Spatiotemporal control of signalling on the KMN network. Schematic to describe how a network of kinases and phosphatases regulate the SAC and kinetochore-microtubule attachments. **(A)** In prophase, the kinase activities of MPS1 and Aurora B are unopposed and key signalling events are initiated. **(B)** In prometaphase, on unattached kinetochores, PP2A-B56 is recruited via BUBR1, which begins to antagonise Aurora B to recruit PP1 to KNL1. PP2A-B56 also antagonises Aurora B to promote kinetochore-microtubule attachments, whereas PP1 antagonises MPS1 to limit KNL1-MELT phosphorylation and prime the SAC for rapid silencing. **(C)** Following microtubule attachment at metaphase, PP1 reverses KNL1-MELT phosphorylation and antagonises Aurora B to stabilise kinetochore fibres.

Figure 2. The different types of kinetochore-microtubule attachments regulated during error-correction. (A) A zone of Aurora B activity encompasses unattached kinetochores to destabilise any kinetochore-microtubule attachments that form. **(B)** Following bipolar attachment, tension across the kinetochore stabilises microtubules by restricting Aurora B from dephosphorylating key kinetochore substrates. **(C, D, E)** The various types of microtubule attachments that do not generate sufficient tension and can therefore be destabilised by Aurora B.

Figure 3. Cross-talk between kinases and phosphatases at the kinetochore. (A) A positive feedback loop that controls MPS1 and Aurora B localisation. **(B)** A negative feedback loop that controls PP1 and PP2A-B56 localisation. **(C)** Cross-talk between both kinases and both phosphatases at the kinetochore. See text for full details of this cross-talk.

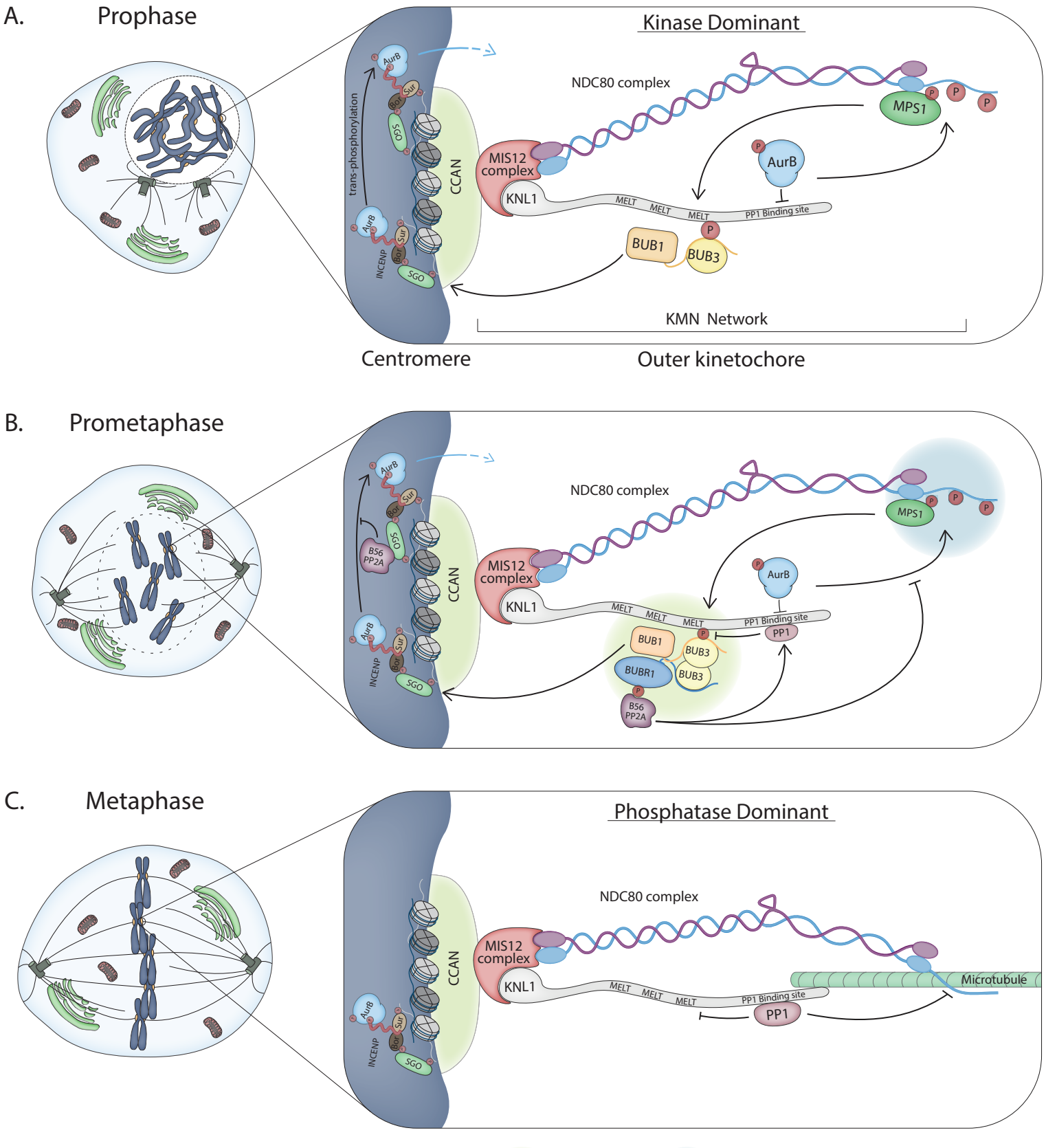
A.
Prophase
Kinase Dominant
NDC80 complex
MPS1
AurB
MIS12 complex
KNL1
MELT
MELT
MELT
PP1 Binding site
BUB1
BUB3
trans-phosphorylation
CCAN
INCENP
Bor
Sur
SGO
KMN Network
Centromere
Outer kinetochore
B.
Prometaphase
B56 PP2A
PP1
BUBR1
C.
Metaphase
Phosphatase Dominant
Microtubule
SAC
Kinetochore-Microtubule Attachments

Figure 1

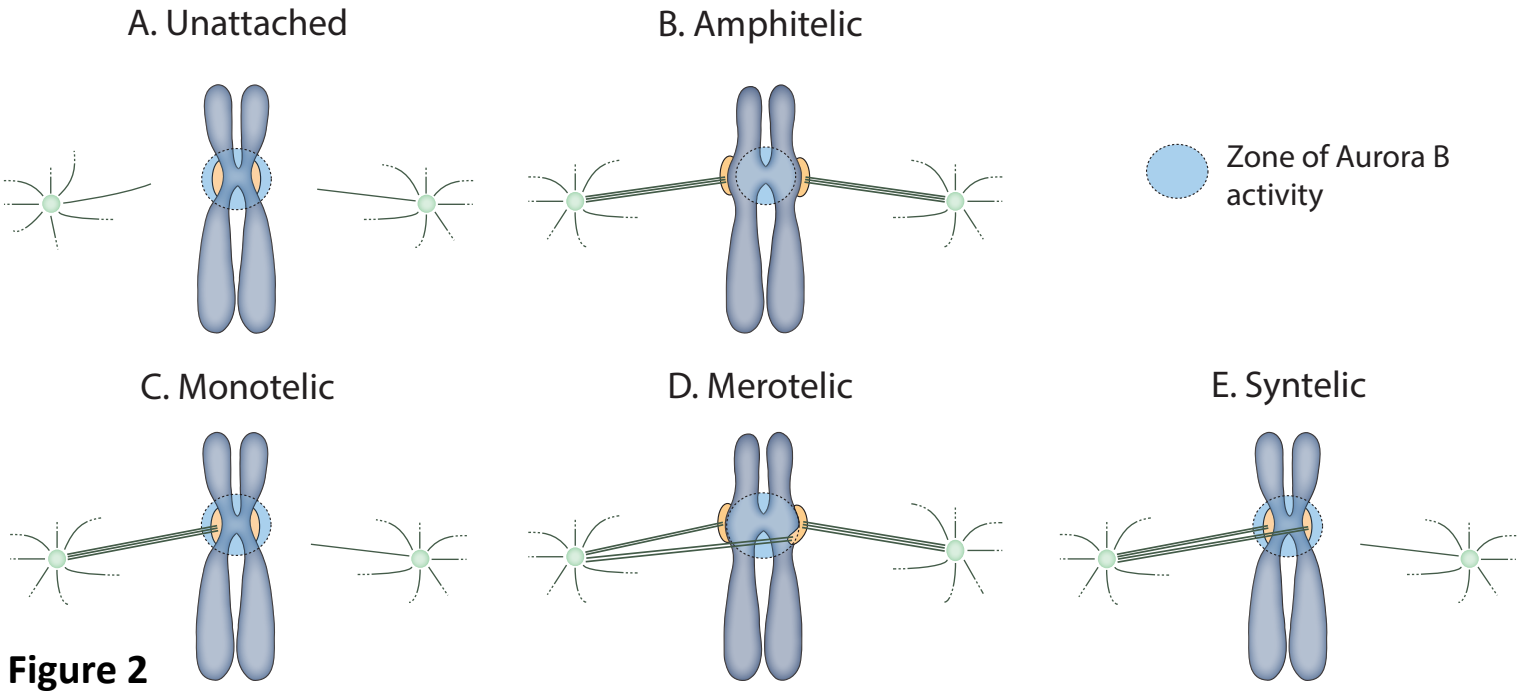
A. Unattached
B. Amphitelic
Zone of Aurora B activity
C. Monotelic
D. Merotelic
E. Syntelic

Figure 2

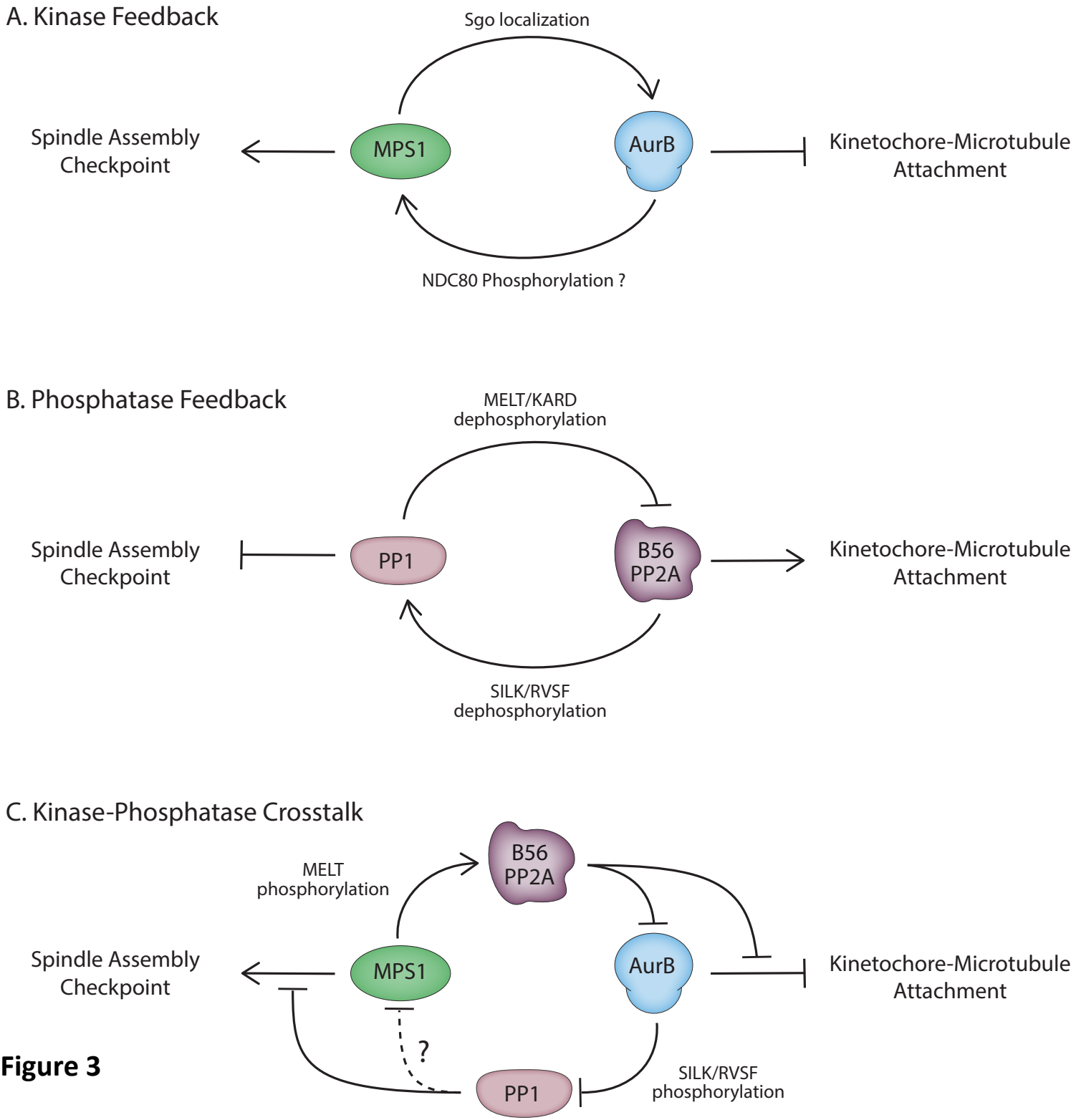
A. Kinase Feedback
Sgo localization
Spindle Assembly Checkpoint
MPS1
AurB
Kinetochore-Microtubule Attachment
NDC80 Phosphorylation ?
B. Phosphatase Feedback
MELT/KARD dephosphorylation
Spindle Assembly Checkpoint
PP1
B56 PP2A
Kinetochore-Microtubule Attachment
SILK/RVSF dephosphorylation
C. Kinase-Phosphatase Crosstalk
B56 PP2A
MELT phosphorylation
Spindle Assembly Checkpoint
MPS1
AurB
Kinetochore-Microtubule Attachment
?
PP1
SILK/RVSF phosphorylation

Figure 3